ANTI-PROLIFERATIVE EFFECT OF METHYL GALLATE ISOLATED FROM *M. Pajang* Kosterman IN SELECTED CANCER CELL LINES

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Adherence, effort and dedication were the fundamental elements for the completion of my master dissertation, but even more was the support from my family members, to both of my parents Che Rahim bin Che Mat, Rohani binti Abd Rahman, to my dearest husband, Muhammad Fawwaz Haikal bin Fauzi, my siblings, Mohd Taufiq, Nabihah and Adam, today I dedicate them this important achievement, because without their presence, support and comprehension, I would have not achieve what I have today.

I also dedicate this to:

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ABSTRACT

Mangifera pajang (Bambangan) is an indigenous fruit originated from Borneo Island. The kernel of *M. pajang* has been reported to possess various health benefits due to the phytochemicals content. As cancer is one of the most leading cause of death in Malaysia, this study was aimed to isolate and elucidate an aromatic ester, methyl gallate from the kernel extract of the *M. pajang* and determine the anti-proliferative potential of aromatic ester, methyl gallate which induce the cell cycle arrest and killing mechanism via apoptosis in selected cancer cell line. Chromatography methods were used in this study to isolate methyl gallate ($C_8H_8O_5$) from methanol crude extract. Identification of the isolated compound was done by spectroscopic methods including infrared (IR), mass spectrometry (MS), nuclear magnetic resonance (NMR) and comparison with reported data. The potential of anti-proliferative activity was investigated by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay against breast (MCF-7 and MDA-MB-231), prostate (PC-3), pancreatic (Capan-2) and colon (HCT-116) cancer cell lines. The effect of methyl gallate on cell cycle arrest and apoptosis were confirmed by using cell cycle analysis and Annexin V staining. The results showed that methyl gallate displayed significant anti-proliferative activity against MCF-7 and moderate activity against PC-3 with an IC_{50} values of 54.7 µM and 97.6 µM respectively. Based on the IC_{50} value of MTT assay, MCF-7 cell line was selected for further determination of cell cycle arrest and apoptosis analysis. The cell cycle progression of MCF-7 cells treated with methyl gallate showed increased in cell population in sub-G_1 phase indicating apoptosis and cell cycle arrest at G_0/G_1. In addition, the effect of methyl gallate on apoptosis induction resulted in total apoptotic cells were increased in time dependent manner. As a conclusion, methyl gallate isolated from kernel of *M. pajang* possess antiproliferative effect against breast cancer cell via apoptosis pathway.

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ABSTRAK

Mangifera pajang (Bambangan) ialah buah-buahan nadir yang berasal dari kepulauan Boneo. Biji buah *M pajang* dilaporkan mempunyai pelbagai manfaat terhadap kesihatan dan ini adalah disebabkan kehadiran pelbagai fitokimia. Memandangkan kanser merupakan penyebab utama kematian di Malaysia, objektif kajian ini adalah untuk memencilkan sebatian ester aromatik, metil gallat ($C_8H_8O_5$) daripada ekstrak biji buah *M. pajang* dan menentukan potensi aktiviti anti-proliferatif yang telah mengaruhkan mekanisma pembunuhan sel secara terkawal (apoptosis) dan fasa-fasa sel. Kaedah kromatografi yang digunakan telah berjaya memencilkan ester aromatik, metil gallat ($C_8H_8O_5$) daripada ekstrak mentah metanol. Pengenalpastian sebatian dilakukan dengan menggunakan kaedah spektroskopi termasuk inframerah (IR), spektroskopi jisim (MS), resonans magnetik nuklear (NMR) dan perbandingan data yang sedia ada. Potensi aktiviti anti-proliferatif oleh sebatian pencilan telah dilakukan dengan menggunakan asai MTT terhadap beberapa jenis sel kanser seperti kanser payudara (MCF-7 dan MDA-MB-231), prostat (PC-3), pankreas (Capan-2), dan kolon (HCT-116). Analisis pembunuhan sel yang dirawat dengan metil gallat terhadap kematian secara apoptosis dan fasa-fasa sel dilakukan dengan analisis fasa sel dan pewarnaan Annexin V. Keputusan aktiviti sitotoksik oleh metil gallat menunjukkan aktiviti yang signifikan terhadap sel kanser payudara (MCF-7) dengan dengan nilai IC_{50} 54.7 µM dan aktiviti sederhana terhadap sel kanser prostat (PC-3) dengan nilai IC_{50} 97.6 µM. Berdasarkan keputusan asai MTT, sel kanser payudara (MCF-7) telah dipilih untuk kajian mekanisma pembunuhan sel dan fasa sel. MCF-7 sel yang dirawat dengan metil gallat menunjukkan peningkatan peratusan sel dalam fasa sub-G_0 dan terdapat penahanan sel berlaku pada fasa G_0/G_1. Manakala, keputusan asai apoptosis menunjukkan sel yang dirawat dengan metil gallat mendorong kematian secara selari dengan masa rawatan. Sebagai rumusan, sebatian terpencil daripada biji buah *M. pajang*, metil gallat mempunyai kesan sebagai bahan anti-proliferatif ke atas sel barah payudara.

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LIST OF SYMBOLS AND ABBREVIATIONS

°C	-	Celcius
cm	-	Centimeter
g	-	Gram
h	-	Hour
IC_{50}	-	Concentration that needed to produce 50% cells inhibition
L	-	Litre
M	-	Molar
mg	-	miligram
mL	-	mililitre
mm	-	milimeter
mM	-	miliMolar
nm	-	nanometer
µg	-	microgram
µL	-	microlitre
µM	-	micro molar
ATCC	-	American Type Culture Collection
CC	-	Column chromatography
CDK	-	Cyclin Dependent Kinase
DIP	-	Direct Induction Probe
DNA	-	Deoxyribonucleic acid
DPPH	-	2,2-diphenyl-1-pycrylhydrazyl radical
EI-MS	-	Electron Impact-Mass Spectrometry
FBS	-	Fetal Bovine Serum
FRAP	-	Ferric reducing/ antioxidant power
FT-IR	-	Fourier Transform-Infrared
G_0	-	Resting phase

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G_1	-	Gap 1 phase
G_2	-	Gap 2 phase
HCT-116	-	Colon Cancer cell lines
MCF-7	-	Hormone dependent breast carcinoma cell line
MCF-10A	-	Normal human breast cell
MDA-MB-231	-	Non-hormone dependent breast carcinoma cell line
MHz	-	MegaHertz
MOH	-	Ministry of Health
MTT	-	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NMR	-	Nuclear Magnetic Resonance
PC-3	-	Prostate carcinoma cell line
PI	-	Propidium Iodide
RNase	-	Ribonuclease
RPMI 1640	-	Roswell Park Memorial Institute 1640
S	-	Synthesis phase
TFC	-	Total Flavonoid Content
TLC	-	Thin Layer Chromatography
TPC	-	Total Phenolic Content
WHO	-	World Health Organization

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LIST OF PUBLICATIONS

Journals:

(i) **Azlen Che Rahim,** Mohd Fadzelly Abu Bakar, Nur Kartinee Kassim, Johnson Stanslas, Wan Nor Izzah Wan Mohamad Zain. (2018). Selective cytotoxic activity of methyl 3,4,5-trihydroxybenxoate isolated from Kernel of Bambangan (*Mangifera pajang*). *Asian Journal of Chemistry, 30*(10): 2273-2276.

(ii) **Azlen Che Rahim**, Mohd Fadzelly Abu Bakar, Mur Kartinee Kassim, Johnson Stanslas. Methyl Gallate isolated from *Mangifera pajang* display anti-cancer activity by induction of cell arrest and apoptosis in human breast cancer cell. [Submitted to *Food and Function*]

Conference:

(i) International Conference on Drug Discovery and Translational Medicine (ICDDTM 2018), 4-5 December 2018, The Everly Hotel, Putrajaya Malaysia.

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CHAPTER 1

INTRODUCTION

1.1 Background of Research

Cancer is a major public health burden worldwide and it is the leading cause of death in both developed and developing countries. The increasing number of death due to cancer is correlated with the modernisation of lifestyle, improved socioeconomic status (Asegaonkar *et al*., 2015), and exposure to sunlight (Zima *et al*., 2001). According to a report by Torre *et al.* (2015), high cancer incidences including lung, colorectal, breast, and prostate cancer are reported to occur at high income countries (HIC). Meanwhile, cancer incidence for low and middle-income countries (LMIC) is growing, with stomach, liver, oesophageal, and cervical cancer having the highest rate. It is also estimated that over 20 million of new cases will be expected annually by as early as 2025 (Bray, 2014). According to an online database of The International Agency for Research on Cancer (IARC)- GLOBOCAN 2012, prostate and lung cancer are top cases commonly diagnosed among males in 87 countries worldwide, while breast and cervical cancers are the most frequently diagnosed in females (Torre *et al.*, 2015).

Chemoprevention has become familiar in the cancer research area, particularly in drug development, pharmacology, and molecular biology research. People has realised that effective cancer treatments such as chemotherapy, radiotherapy, and drug therapy still could not challenge the potential of chemoprevention as the survival rate of cancer patients is still low (Ladas, 2004; Liu, 2009). Chemoprevention is an alternative in preventing the development of cancer by using specific agents either to inhibit, delay, or reverse carcinogenesis (Tamimi *et al*., 2002). Meanwhile, chemotherapy is another term that is involved in the cancer research. Unlike the

chemoprevention, chemotherapy is one of modes of treatment in cancer patients with the utilisation of chemotherapeutic drugs (Liu, 2009).

Malaysia is one of the Asian countries that are blessed with diverse biological resources and high in percentage of flora, which are believed to have medicinal and nutritional values. It is estimated that there are about 370 species of local fruits in Malaysia (Rukayah, 2002; Mirfat, Salma, & Razali, 2016). Other than for commercialisation purposes, tropical fruits in Malaysia are grown for local consumption and medicinal uses. Most of the indigenous fruits in Malaysia are wild fruits that are naturally distributed in the forests and most of them are underutilised (Abu Bakar & Fry, 2013). Some of the underutilised fruits especially in Borneo tha t are available in Malaysia are bacang (*Mangifera foetida*), bambangan (*Mangifera pajang*), cerapu (*Garcinia prainiana*), durian (*Durio*), jambu (*Syzygium aqueum*), kuini (*Mangifera odorata*), pulasan (*Nephelium mutabile*), and salak (*Salacca zalacca*) (Hock *et al.*, 2016). Many of these fruits are unpopular due to the lack of promotion, not being fully explored, and minimal space for planting (Chai *et al.*, 2008).

The nutritional and medicinal benefits of plants, fruits, and vegetables are receiving great attention nowadays as they have been shown to provide a better support for human wellbeing. Recently, plant-derived products have been the major target for many studies due to their versatile applications. Some medicinal plants have been reported to possess anticancer activity, where polyphenols from fruits and vegetables are the responsible molecules for chemopreventive effects (Mutalib *et al.*, 2016). It has also been reported that plant-derived materials possess highly diverse and complex molecular structures compared to synthetic drugs and play an important role in human health and in the development of new anticancer drugs (Faezizadeh, Gharib, & Godarzee, 2016). A study by Liu (2004) also reported that diets which are rich in fruits and vegetables are good sources of natural phytochemicals which work synergistically to exhibit antioxidant and anticancer activities.

Phytochemicals in plants play a major role in contributing to human health. High dietary intake of fruits and vegetables as well as whole grains is strongly associated with reduced risk of getting chronic diseases, such as cancer and cardiovascular diseases (CVD). The United States National Academy of Sciences also emphasised the importance of adding citrus fruits, carotene-rich fruits, and vegetables to diet to reduce the risk of cancer (Liu, 2003). Research and development of rare and underutilised fruits is becoming economically important nowadays as it promotes a

new generation of ‘superfruits’ which have the potential to be developed as functional food and nutraceuticals.

In Southeast Asia, there are many varieties of local fruits such as mango (*Mangifera indica*), durian (*Durio*), rambutan (*Nephelium lappaceum*), and papaya (*Carica papaya*), which are sold locally and globally. However, there are also fruits that are sold locally but have not been fully explored. For example, *Mangifera pajang* is a native fruit that can only be found in the Borneo Islands (Malaysia: Sabah, Sarawak; Brunei; Indonesia: Kalimantan) and is rarely discovered in Peninsular Malaysia. This fruit is prevalent among the Kadazan-Dusun community in Sabah as an accompaniment to their meal and traditional cuisine (Tangah *et al.*, 2017). Many studies on *M. pajang* phytochemicals have reported their health-beneficial properties.

1.2 Problem statement

Based on cancer statistics, deaths caused by cancers are known to increase annually. The leading cancers among males in Malaysia are lung, nasopharynx, stomach, urinary bladder, rectum, liver, and colon cancers. Meanwhile, cervix, breast, lung, colon, and rectum cancers are majorly being diagnosed in females in Malaysia (Kasri, 1993). In addition, Azizah *et al.* (2016) added that Malay females have the highest rate of breast cancer incidence. The increase in death rate due to cancer are related to several factors such as burden to patients due to the increase in cost for cancer treatment. Not only that, current cancer therapies including surgery, radiotherapy, chemotherapy, and drugs have a major drawback towards the patients such as bad side effects after the therapy (Partridge *et al.*, 2001). The cancer patients must bear with symptoms such as nausea, anaemia, and hair loss. Not only that, the development of chemoresistance is a persistent problem and makes it difficult for the patients to deal with the treatment. There is thus a great need towards finding a better alternative on cancer therapy including traditional medicine, which has become an important approach to control cancer. Apart from that, there has been a focus on natural products from plant sources to be developed as functional food to provide better ways for cancer patients to reduce their burden.

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1.3 Objectives of the study

General objective:

To explore the potential of local fruits, *Mangifera pajang* to be developed as nutritional food.

Specific objectives:

(i) To isolate and elucidate methyl gallate from the kernel of *M. pajang*.

(ii) To evaluate the *in-vitro* anti-proliferative activity of the methyl gallate extracted from kernel extract of *M. pajang* against selected cancer cell lines

(iii) To evaluate the effect of methyl gallate isolated from kernel extract of *M. pajang* on apoptosis event and cell cycle arrest in selected cancer cell lines.

1.4 Significance of study

With the increasing trend of cancer incidence of each year, number of efforts are needed in order to provide better alternative to reduce the percentage of mortality due to cancer. One of the efforts is through the changes in diet (Willet, 1995). Diet that is rich in plants and fruits could be a better solution in reducing cancer incidence as fruits and plants are known to possess various health benefits and anticancer properties. Fruits and vegetables play a vital role in the health of human beings by providing various phytochemicals such as phenolic compounds and flavonoids to promote health (Abu Bakar *et al.*, 2010). These compounds are known to be natural antioxidants which are abundantly present in plants and fruits. These compounds have been the main target for scientists as they provide not only nutritional value but have promising effects on health as well.

Since many studies have provided findings on the biological and health-promoting effects of *M. pajang*, further evaluation of anticancer properties of the active extract from this fruit can provide more details about the active compounds and their biological activities. With the aim to identify and isolate active natural compounds from the active extract of *M. pajang* fruit, this study provides a better explanation in the fundamental mechanism of actions in killing cancer cells.

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1.5 Scope of study

In the present study, the antiproliferative potentials of natural products were explored. *M. pajang* fruit is abundantly found in Borneo Island and the focus was given on its kernel part. Given that many past studies have reported that *M. pajang* kernel displayed many health benefits such as antioxidant activity due to the diversity of polyphenols. Higher antioxidant activity has suggested by some in-vitro and in-vivo study which certain antioxidant agent selectively inhibit the growth of tumour cells, induce the cellular differentiation, and also may alter the intracellular redox state, hence enhances the effect of cytotoxic therapy (Ladas *et al.*, 2004). In this study, emphases are given to the one of the remarks on the cancer manifestation that involved in the cancer development, which is apoptosis. The effects of the natural products on cancer development are also discussed.

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CHAPTER 2

LITERATURE REVIEW

2.1 Cancer

Cancer is a disease where abnormal cells continue to grow in an uncontrolled manner and disregard the normal rules of cell division (Kumar *et al*., 2016). Normally, body cells control the process of cell division, cell death, and cell differentiation, but cancer cells ignore the regulation, resulting in uncontrolled cell growth and proliferation (Li & Wang, 2014). These uncontrolled cancer cells will proliferate, resulting in the formation of tumours (Kumar *et al*., 2016). This abnormal proliferation can continue and spread to other parts of the body; this is called metastasis and may be fatal (Hejmadi, 2009).

There are two types of cancer cells, which are malignant and benign tumour cells. Benign tumours are tumours that do not spread and usually pose little threat to the host since they are localised and small (Lodish, Berk, & Zipursky, 2000). On the other hand, malignant tumours are non-localised and invade other surrounding tissues (Lodish *et al*., 2000). A benign tumour has a slower growth speed than a malignant one (Baba & Catoi, 2007).

Cancer can be treated in several ways; the available technologies today are surgery, radiotherapy, immunotherapy, and chemotherapy. Some of the methods use anticancer drugs or defined as chemopreventive agents, such as those derived from plants such as taxol (found in *Taxus brevifolia*), combretastatin A-4 (from *Combretum caffrum*) (Srivastava *et al.*, 2005), camptothecin (isolated from *Camptotheca acuminata*), vinblastine, and vincristine (found in *Catharanthus roseus*) (Colgate & Molyneux, 2007).

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2.1.1 Apoptosis and cell cycle

Apoptosis is a programmed, physiological mode of cell death mechanism for removing unwanted and detrimental cells in a silent manner during embryonic development, tissue homeostasis, and immune regulation (Orangi *et al.*, 2016). The normal regulation of this molecular mechanism becomes a drawback when the pathway of apoptotic signalling is altered, leading to development of cancer and diseases (Favaloro *et al*., 2012). Cells that have severe DNA damage and are unsuccessfully removed by apoptosis would acquire mutation and this enables them to grow and proliferate uncontrollably.

Apoptosis can be initiated by two pathways: extrinsic and intrinsic. The extrinsic pathway is induced by extracellular stimuli such as binding of ligand to death receptor on cell surface or cytokine stimulation which results in the activation of caspase cascade and alteration in gene expression profile. Meanwhile, the intrinsic pathway is induced by many ranges of stimuli such as DNA damage, transcription/translation damage, or virus infection (Hollier *et al.*, 2007).

Caspase-dependent apoptosis is characterised by the activation of a series of pathways, leading to the activation of a family of proteases (caspases), resulting in an ordered disruption, which is a more favourable way of cell disruption without the leakage of cellular components and induction of inflammation (Favaloro *et al*., 2012).

Apoptosis is more favourable than necrosis due to the response of the cells towards receiving the cell-death signals. A report by Saraste & Pulkki (2000) briefly described the characteristics of cell at the onset of apoptosis: it begins with the shrinkage of the cell and the nucleus, followed by condensation of nuclear chromatin masses. Next, the nucleus progressively breaks up; this is termed as karyorrhexis. The budding cells are detached from the surrounding tissue and become extensions and the plasma membrane seals to form a separate membrane around detached solid cellular materials, or known as apoptotic bodies, which are enclosed together with cellular organelles and fragments of the nucleus. Later, these apoptotic bodies are engulfed by neighbouring cells such as macrophages and parenchyma cells (Kerr *et al.*, 1994).

In a cell cycle research, it is necessary to synchronise a population of cells at a stage in the cell cycle, so that the cellular or biochemical features of that stage can be analysed. Numerous drugs, including thymidine and hydroxyurea, block DNA synthesis by inhibiting the synthesis of specific nucleotides, which results in a

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